*Communication*



# Identification in the Human Central Nervous System, Pituitary, and Thyroid of a Novel Calcitonin Gene-related Peptide, and Partial Amino Acid Sequence in the Spinal Cord*

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Two human genes encoding precursors for two calcitonin gene-related peptides (CGRP) I (or α) and II (or β) have been identified (Steenbergh, P. H., Höppener, J. W. M., Zandberg, J., Lips, C. J. M., and Jansz, H. S. (1985) *FEBS Lett.* 183, 403–407). The amino acid sequence of CGRP-I was obtained in medullary thyroid carcinoma extracts (Morris, H. R., Panico, M., Etienne, T., Tippins, J., Girgis, S. I., and MacIntyre, I. (1984) *Nature* 308, 746–748), but not in normal human tissues. The human CGRP-II peptide remained to be discovered. Here we have determined in the human spinal cord the amino acid composition and the partial amino acid sequence of the DNA-predicted CGRP-I and -II. The data indicate for the first time the existence of a second CGRP different from the known CGRP-I. CGRP-II has been identified in the central nervous system, pituitary, thyroid, and in medullary thyroid carcinoma as a major CGRP form together with CGRP-I.

Processing of calcitonin gene transcripts results in the formation of CGRP[1] predominantly in neural tissue, whereas calcitonin is the major gene product in the C-cells of normal thyroid glands and in medullary thyroid carcinoma (1–7). In normal human tissues, the amount of CGRP coeluting with synthetic human CGRP-I (or α)[2] on high performance liquid chromatography (HPLC) was higher when estimated by radioreceptor assay than by radioimmunoassay (6). We therefore concluded that the radioreceptor assay recognized an additional CGRP-like component different from human CGRP-I. Analysis of DNA complementary to mRNAs from

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[1] The abbreviations used are: CGRP, calcitonin gene-related peptide; HPLC, high performance liquid chromatography; RIA, radioimmunoassay.

[2] CGRP-I and α, and CGRP-II and β, are synonyms.

human and rat medullary thyroid carcinoma revealed sequences encoding a second CGRP (or β) in human and rat (8–10). Corresponding mRNA has been recognized in the rat brain and thyroid gland (9), but so far not in normal human tissues. Comparison of the DNA-predicted amino acid sequence of rat and human CGRP-I and -II reveals that the peptides differ by one and three amino acids, respectively (9, 10). Human CGRP-I has been sequenced in extracts of medullary thyroid carcinoma (11) and in the tissue culture medium of a human medullary thyroid carcinoma cell line.[3] The existence of the second CGRP peptide remained to be ascertained.

Here we have isolated CGRP-I and -II for the first time from a normal tissue, the human spinal cord. The amino acid composition and partial amino acid sequence of the peptides are consistent with the DNA-predicted sequences (10) and reveal the presence of CGRP-I and -II in a neural tissue. Moreover, both peptides have been identified in the human brain, in the pituitary, in the thyroid gland, and in medullary thyroid carcinoma.

## MATERIALS AND METHODS

*Peptides and Tissues*—Synthetic CGRP-I and -II were purchased from Peninsula Laboratories (Belmont, CA) and were shown to be more than 90% pure on reverse-phase HPLC using an acetonitrile gradient (21).

Spinal cords (5–16 g), cerebellar cortex (9–16 g), thalamus (2–4 g), pituitary glands (0.4–0.6 g), thyroid glands (7–15 g), and medullary thyroid carcinoma and metastases thereof (1–10 g) have been obtained at autopsy not later than 20-h postmortem and frozen at −80 °C. Medullary thyroid carcinoma and metastases thereof were also obtained at surgery and frozen within minutes on dry ice. TT human medullary carcinoma cells were grown as monolayers in RPMI 1640 supplemented with 16% heat-inactivated fetal calf serum and 3.5 g/liter of $NaHCO_3$ at 37 °C in an 8% $CO_2$ atmosphere as described (22).

*Extraction, Purification, and Isolation*—14–26-g aliquots of spinal cords were extracted using Sep-Pak $C_{18}$ cartridges (Waters) and purified by reverse-phase HPLC on Nucleosil 10 $C_{18}$ (Macherey-Nagel) columns with an acetonitrile gradient according to previously described methods (Fig. 1A) (4, 21). Thereafter, partially purified immunoreactive CGRP (0.5–1.0 nmol, equivalent to 14–26 g of wet spinal cords) in effluent fractions with the retention times of synthetic CGRP-I and -II were subjected to cation-exchange chromatography on a TSK SP-5-PW (Bio-Rad) column (75 × 7.5 mm) (Fig. 1B). The column was equilibrated with 20 mM sodium phosphate, pH 8.4, and 40% acetonitrile (Fluka), and the material was dissolved and injected into the column in 2 ml of the same buffer. The column was eluted at room temperature with a 30-min gradient of 0–0.5 M NaCl in the same buffer. The flow rate was 0.8 ml/min, and 0.8-ml fractions were collected. Effluent fractions containing peaks with immunological characteristics of CGRP-I and -II (see below) and the retention times of the two synthetic peptides were lyophilized. CGRP-I and -II (0.1–0.5 nmol, equivalent to 14–26 g of wet spinal cord) were rechromatographed using reverse-phase HPLC on a $C_{18}$ column described in Fig. 1A, and effluent fractions with the retention times of CGRP-I and -II were lyophilized. Purified CGRP-I and -II (0.8 and 1.4 nmol, respectively, equivalent to 260 g of wet spinal cord) were dissolved in 2 ml of 0.1 M trifluoroacetic acid containing 20% acetonitrile and injected in a $C_{18}$ column equilibrated with the same buffer. Highly purified CGRP-I and -II were eluted at room temperature with a 75-min gradient of 20–50% acetonitrile in 0.1% trifluoroacetic acid. The flow rate was 1.5 ml/min, and 1.5-ml fractions were collected (Fig. 1, C and D). Immunoreactive CGRP was analyzed by radioimmunoassays recognizing predominantly CGRP-I and -II (see below), and

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[3] J. B. Petermann, W. Born, H. R. Morris, B. A. Roos, and J. A. Fischer, manuscript in preparation.

proteins were determined at 210 nm. The lyophilized CGRP-I and -II (1.2 nmol of CGRP-I and 0.7 nmol of CGRP-II) were subjected to analysis of amino acid composition and sequenced (Tables I and II).

Identification of CGRP-I and -II in the remaining tissues was carried out after extraction with Sep-Pak $C_{18}$ cartridges (21) by a combination of cation-exchange HPLC (see above) and radioimmunoassays recognizing predominantly CGRP-I and -II (Fig. 2, Table III).

*Amino Acid Composition and Sequence Analysis*—The amino acid composition of purified CGRP-I and -II from the spinal cord was performed with the dimethylaminoazobenzene sulfonyl chloride precolumn derivatization method (23, 24). CGRP-I and CGRP-II were hydrolyzed by the gas phase in 6 N HCl and dabsylated, and 5% were injected for analysis.

Amino acid sequence analysis of CGRP-I and -II was done by automated Edman degradation using a gas/liquid phase microsequenator (Applied Biosystems) (25). Phenylthiohydantoin-derivatives of amino acids were analyzed by HPLC (26). Manual Edman degradation was carried out by the 4-(dimethylamino)azobenzene 4'-isothiocyanate/phenyl isothiocyanate double-coupling method (27). Cysteic acid was identified as the dimethylaminoazobenzene thiohydantoin-derivative by HPLC on a reverse-phase $C_{18}$ (5-μm) column (Brownlee), using a modification of published conditions (27).

*Radioimmunoassay (RIA)*—CGRP in tissue extracts and column effluent fractions was determined as described previously (6, 28). CGRP-I was analyzed in a homologous RIA which recognized CGRP-I with a half-maximal inhibition of the immunological reaction of 30 pM which was 20 times lower than that of CGRP-II. In contrast, antibodies raised to CGRP-II recognized the peptide with a half-maximal inhibition of the immunological reaction of 90 pM which was 70-fold lower than that of CGRP-I. In both CGRP RIAs, synthetic human calcitonin, corticotropin, α-melanotropin (Ciba-Geigy), salmon calcitonin (Sandoz), angiotensin I, β-endorphin, vasopressin, oxytocin, substance P (Beckman), [Leu⁵]- and [Met⁵]enkephalin (Bachem), and the carboxyl-terminal flanking peptide of calcitonin, PDN-21 (Peninsula Laboratories, Inc.) were not detected in amounts of as high as 20 μg/ml. Immunodilution curves of synthetic CGRP-I and -II were parallel to those obtained with the extracted peptides.

## RESULTS AND DISCUSSION

Here we report for the first time the presence of a CGRP different from CGRP-I. The amino acid sequence of CGRP-I has been obtained from extracts of medullary carcinoma of the thyroid (11) but not from a normal human tissue such as the spinal cord. Precursor processing and certain CGRP- and calcitonin-like peptides are not necessarily the same in malignant tumors as in tissues subjected to physiological regulation (12, 13).

The CGRP-I and -II have been extracted from 22 spinal cord samples (260 g) obtained at autopsy and purified to apparent homogeneity (Fig. 1). Single peaks of immunoreactive CGRP-I (1160 pmol) and of CGRP-II (710 pmol) have been obtained on HPLC with an overall yield of 5 to 15%. Human CGRP-I and -II differ by three amino acids (10, 11). The purity of CGRP-I and -II was assessed by comparison of the composition of amino acids with the calculated values from the DNA-predicted sequences (10), and for CGRP-I also with the identical values obtained from the amino acid sequence analysis in medullary thyroid carcinoma (11) (Table I). The presence of 3.7 aspartic acids in CGRP-I (theoretical value 4) and 2.8 (3) in CGRP-II, of a methionine in CGRP-II which is absent in CGRP-I, of 3.1 serines (theoretical value 3) in CGRP-I as compared to 3.8 (4) in CGRP-II, and of 4.1 valines (theoretical value 5) in CGRP-I compared to 3.8 (4) in CGRP-II are consistent with the DNA-predicted (and for CGRP-I with the analyzed) structures of the two peptides. The amounts of immunoreactive CGRP-I and -II subjected to amino acid sequence analysis corresponded to the levels of alanine residues cleaved from the amino terminus during sequencing (Table II). With the limited quantity of pure CGRP-I and -II isolated, only partial, but not complete, amino acid sequence analysis has been accomplished. Therefore,
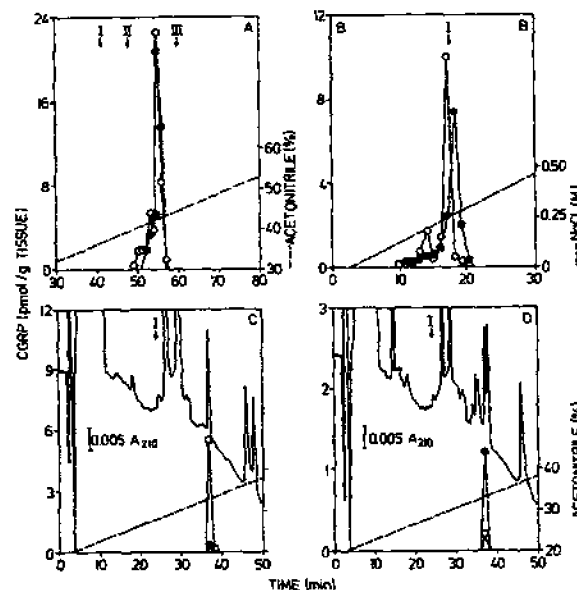


FIG. 1. **Purification of CGRP-I and -II by HPLC from normal human spinal cords.** *A*, reverse-phase HPLC profile of a Sep-Pak $C_{18}$ CGRP extract eluted from a column (Nucleosil 10 $C_{18}$) with a linear gradient of acetonitrile containing heptafluorobutyric acid as hydrophobic anion-pairing reagent (-- --); *B*, cation-exchange HPLC profile of CGRP obtained from the Nucleosil 10 $C_{18}$ column (*A*) and eluted from a column (TSK SP-5-PW) with a linear gradient of NaCl (-- -- --); *C*, reverse-phase HPLC profiles of purified CGRP-I and *D*, of CGRP-II eluted from a Nucleosil 10 $C_{18}$ column with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (-- -- --). Effluent fractions were analyzed for immunoreactive CGRP-I (O) and -II (●), and for protein at 210 nm (——). The *arrows* indicate elution positions of substance P (*I*) (Amersham International), ³H-human calcitonin (*II*) (Ciba-Geigy), and ³H-salmon calcitonin (*III*) (Sandoz) used for calibration.

TABLE I

*Comparison of amino acid composition of CGRP-I and -II from human spinal cord with calculated composition of CGRP-I obtained in medullary thyroid carcinoma (11) and the DNA-predicted sequences of CGRP-I and -II (10)*

The results have been obtained from 2.5 pmol of dabsylated hydrolysates.

| Amino acid | Experimental value | | Theoretical values | |
|---|---|---|---|---|
| | CGRP-I | CGRP-II | CGRP-I | CGRP-II |
| Alanine | 3.8 | 4.3 | 4 | 4 |
| Arginine | 1.7 | 1.6 | 2 | 2 |
| Aspartic acid | 3.7ᵃ | 2.8 | 4 | 3 |
| Cysteine | 1.6ᵇ | 0.6ᵇ | 2 | 2 |
| Glycine | 4.5 | 4.2 | 4 | 4 |
| Histidine | 0.7 | 0.4 | 1 | 1 |
| Isoleucine | 0.2 | 0.3 | 0 | 0 |
| Leucine | 3.4 | 3.4 | 3 | 3 |
| Lysine | 2.2 | 2.7 | 2 | 2 |
| Methionine | 0 | 0.9 | 0 | 1 |
| Phenylalanine | 2.2 | 1.6 | 2 | 2 |
| Proline | 1.4 | 1.8 | 1 | 1 |
| Serine | 3.1 | 3.8 | 3 | 4 |
| Threonine | 3.5 | 2.5 | 4 | 4 |
| Tyrosine | 0.5 | 0.3 | 0 | 0 |
| Valine | 4.1 | 3.8 | 5 | 4 |

ᵃ The underlined amino acids are different in CGRP-I and -II.
ᵇ Cysteines are identified as Cys-Cys and multiplied by 2.

sequencing was carried out up to amino acid residue 35 (out of 37) for CGRP-I and up to residue 28 for CGRP-II. All amino acid residues identified in the CGRP-I and -II are in exact agreement with the DNA-predicted (and for CGRP-I

TABLE II

*Amino acid alignment of the sequenced CGRP-I and -II from human spinal cord with the amino acid sequence of CGRP-I obtained in medullary thyroid carcinoma (11), and the DNA-predicted sequences of CGRP-I and -II (10)*

The results have been obtained by automated Edman degradation from 520 pmol of CGRP-I and 430 pmol of CGRP-II. The underlined amino acids are different in CGRP-I and -II. —, identical. NQ, not quantified.

Residues 1–13:

| | | 1 | | | | | | | | | 10 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Predicted sequence | CGRP-I | Ala | Cys | Asp | Thr | Ala | Thr | Cys | Val | Thr | His | Arg | Leu | Ala |
| Sequence data | | — | — | Asp | — | — | — | X | — | — | — | — | — | — |
| Yield (pmol) | | 595 | a | 370 | NQ | 347 | NQ | | 432 | NQ | NQ | NQ | 187 | 157 |
| Predicted sequence | CGRP-II | — | — | Asn | — | — | — | — | X | — | — | — | X | — |
| Sequence data | | — | X | Asn | — | — | — | X | — | — | — | X | — | — |
| Yield (pmol) | | 350 | X | 250 | NQ | 207 | NQ | | 128 | NQ | NQ | | 107 | 98 |

Residues 14–26:

| | | | | | | | | 20 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Predicted sequence | CGRP-I | Gly | Leu | Leu | Ser | Arg | Ser | Gly | Gly | Val | Val | Lys | Asn | Asn |
| Sequence data | | — | — | — | — | — | — | X | — | Val | — | — | Asn | — |
| Yield (pmol) | | 135 | 187 | 265 | NQ | NQ | NQ | | 115 | 95 | 160 | 65 | 38 | 68 |
| Predicted sequence | CGRP-II | — | — | — | — | — | — | — | — | Met | — | — | Ser | — |
| Sequence data | | — | — | — | — | X | — | — | — | Met | — | — | Ser[b] | — |
| Yield (pmol) | | 83 | 105 | 150 | NQ | | NQ | 50 | 73 | NQ | 25 | 35 | | 8 |

Residues 27–37:

| | | | | | 30 | | | | | | 37 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Predicted sequence | CGRP-I | Phe | Val | Pro | Thr | Asn | Val | Gly | Ser | Lys | Ala | Phe·NH$_2$ |
| Sequence data | | — | — | — | X | — | — | — | X | — | X | X |
| Yield (pmol) | | 33 | 80 | 52 | | 25 | 63 | 27 | | 17 | | |
| Predicted sequence | CGRP-II | — | — | — | — | — | — | — | — | — | — | — |
| Sequence data | | — | — | X | X | X | X | X | X | X | X | X |
| Yield (pmol) | | 8 | 30 | | | | | | | | | |

a Cysteine has been identified by manual Edman degradation in 350 pmol of CGRP-I.
b Not asparagine.

TABLE III

*CGRP-I and -II in human tissue extracts*

| Tissue | n | CGRP-I | CGRP-II |
|---|---|---|---|
| | | pmol/g | |
| Spinal cord | 10 | 34.4 ± 6.1[a] | 31.4 ± 5.4 |
| Cerebellar cortex | 3 | 0.027 ± 0.015 | 0.161 ± 0.032 |
| Thalamus | 3 | 0.171 ± 0.098 | 4.27 ± 1.44 |
| Pituitary | 15[b] | 1.6 | 75.7 |
| Thyroid | 3 | 1.51 ± 0.87 | 0.216 ± 0.125 |
| Medullary thyroid carcinoma | 3 | 598 ± 463 | 121 ± 81 |
| TT-medullary thyroid carcinoma cell line | 1 | 41,000 | ND[c] |

a Values reported are mean ± S.E. per g wet weight. For details see Fig. 2.
b Pool.
c ND, not detectable.

with the analyzed) sequences. Together with the amino acid composition data, we present clear evidence for the expression, at the peptide level, of CGRP-I and of a different second CGRP in the human spinal cord.

Besides CGRP-II, another apparently homogenous peak absorbing at 210 nm was noted on Fig. 1D. The later eluting peak was not recognized by antibodies to CGRP-I and -II. Analysis of the sequence of amino acids revealed a structure up to amino acid residue 20 inconsistent with CGRP-I or -II (not shown).

The question then arises as to whether CGRP-II is a minor component or a biologically relevant peptide in relation to CGRP-I. We have isolated CGRP-I and -II from the spinal cord whose dorsal part was known to contain high amounts of CGRP localized in nerve fibers (Fig. 1 and Table III) (6, 14, 15). Other regions of the human central nervous system containing mainly CGRP-II include the cerebellar cortex and the thalamus presenting also CGRP receptors (Fig. 2 and Table III) (6). Among all human tissues examined, the concentrations of immunoreactive CGRP were highest and similar in the spinal cord and the pituitary (6). There, CGRP-II was a major and CGRP-I a minor component (Fig. 2 and Table III).
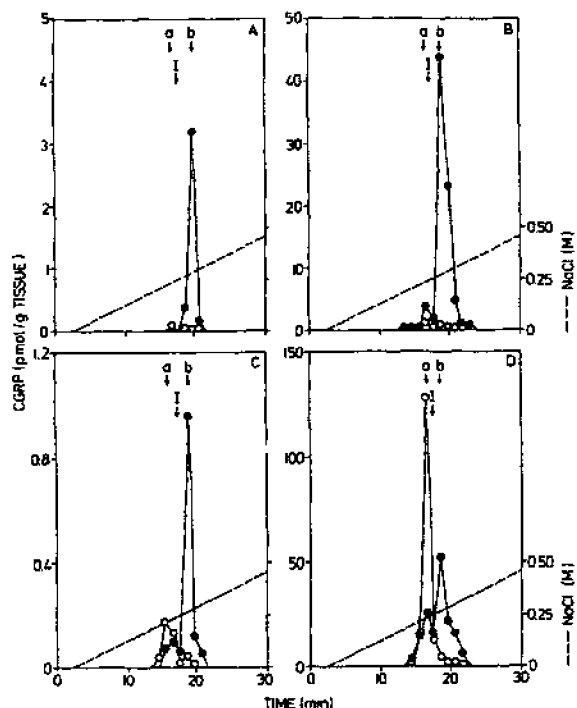
FIG. 2. **Distribution of CGRP-I and -II in human tissues.** Elution profiles of Sep-Pak C$_{18}$ CGRP extracts from columns of TSK SP-5-PW with a linear gradient of NaCl (- - -). Effluent fractions were analyzed for immunoreactive CGRP-I (O) and -II (●). An *arrow* (*I*) indicates the elution position of $^3$H-substance P used for calibration; 10 ng of synthetic human CGRP-I (*a*) and -II (*b*) were analyzed separately. *A*, thalamus; *B*, pituitary; *C*, thyroid; *D*, medullary thyroid carcinoma.

Initially, we had anticipated to isolate CGRP-II from medullary thyroid carcinoma which have been successfully used for the analysis for the structure of CGRP-I and of human calcitonin derived from the same gene (11, 16). Unexpectedly, the amounts of CGRP-II present in medullary thyroid carci-

noma tended to be lower than those of CGRP-I, and CGRP-II was undetectable in cultured medullary thyroid carcinoma (TT) cells. Normal human thyroid glands have been known to contain immunoreactive CGRP (4). Here also both CGRP-I and CGRP-II have been identified.

Little information is available so far on the biological activity of CGRP-II in man and experimental animals. We have shown that CGRP-I and -II, administered to normal human subjects, stimulate the heart rate and cause hypotension and vasodilation concomitant with raised ventricular contractility (29). *In vitro*, CGRP-I and -II are equipotent in the stimulation of the tension of isolated human auricles and in the relaxation of arterial smooth muscle. In man, CGRP-I and -II are more potent in contracting isolated human auricles than norepinephrine, and more potent than acetylcholine in relaxing small arteries. Besides, CGRP-I and -II stimulate the rate and force of contraction of isolated rat atria and cause vasodilatation in rabbit skin (17, 18). The physiological importance of the CGRPs remains to be defined.

The rationale for two human CGRP genes residing on the same chromosome 11 remains to be understood (19). Since CGRP-I and calcitonin are major calcitonin gene products of medullary thyroid carcinoma in man, whereas CGRP-II has been identified as a predominant CGRP component of certain normal human tissues such as the cerebellum, the thalamus, and the pituitary, expression of the two CGRP genes appears to be independently regulated. Calcitonin and CGRP-I are encoded by the same gene. The CGRPs and calcitonin share limited structural homology and may have evolved by gene duplication, and they have related biological effects (for review see Ref. 20). No calcitonin-coding exons have so far been reported in the second human and rat genes (9, 10), but the entire sequence of the second CGRP gene remains to be determined.

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